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Research Article

Identification of differentially expressed genes of *Xanthomonas axonopodis* pv. *citri* by representational difference analysis of cDNA

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Abstract

Xanthomonas axonopodis pv. *citri* is a phytopathogenic bacterium responsible for citrus canker, a serious disease which causes severe losses in citriculture around the world. In this study we report the differential expression of *X. axonopodis* pv. *citri* in response to specific treatments by using Representational Difference Analysis of cDNA (cDNA RDA). cDNAs from *X. axonopodis* pv. *citri* cultured in the presence of leaf extract of the host plant (*Citrus sinensis*), *in vivo*, as well as in the complex medium were hybridized against cDNA of the bacterium grown in the minimal medium. Sequencing of the difference products obtained after the second and third hybridizations revealed a total of 37 distinct genes identified by homology searches in the genome of *X. axonopodis* pv. *citri*. These genes were distributed in different functional categories, including genes that encode hypothetical proteins, genes involved in metabolism, cellular processes and pathogenicity, and mobile genetic elements. Most of these genes are likely related to growth and/or acquisition of nutrients in specific treatments whereas others might be important for the bacterium pathogenicity.

Key words: citrus canker; differential expression, leaf extract, *in vivo*.

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Introduction

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri,* is a serious disease which causes severe losses in the production of citrus in many areas around the world (Rosetti, 1977; Stall and Seymour, 1983). The symptoms include the formation of raised lesions on branches, leaves and fruits.

Several genes involved in pathogenicity have been isolated in xanthomonads, such as the *hrp* (hypersensitive response and pathogenicity), *avr* (avirulence) and *rpf* (regulation of pathogenicity factors) that influence the disease and the severity of the symptoms (Dangl, 1994). Although the major genes involved in the pathogenicity of *X. axonopodis* pv. *citri* are already known, there are very few reports on the general differential expression of this bacterium in the interaction with the host or in environmentally controlled conditions. Most studies have been limited to the analysis of the expression of specific genes (Duan *et al.*, 1999; Swarup *et al.*, 1992).

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The Representational Difference Analysis of cDNA (cDNA RDA) technique has been considered efficient for differential expression studies. This method is based on the subtractive hybridization of two cDNA populations followed by enrichment of the differential products by PCR amplification (Bowler *et al.*, 1999). cDNA RDA was initially developed for eucaryotic cells and has successfully identified several differentially expressed genes in different tissues (Cooper *et al.*, 2000; Kim *et al.*, 2001). In bacteria, cDNA RDA has been rarely used and only a few reports are available (Becker *et al.*, 2001; Bowler *et al.*, 1999).

In the present study we have identified differentially expressed genes of *X. axonopodis* pv. *citri* in response to different growth conditions by cDNA RDA. The modified MM1 medium (Schulte and Bonas, 1992), described in the induction of the *hrp* genes in *X. campestris*, was used as a basal medium. cDNA from the bacterium grown in the presence of leaf extract from a susceptible host (sweet orange) as well as in the host plant leaves and complex medium was used in successive rounds of subtractive hybridizations against cDNA from the MM1 treatment.

Materials and Methods

Bacterial strains and culture conditions

X. axonopodis pv. *citri* 306, obtained from the culture collection of plant pathogenic bacteria of IAPAR (Instituto Agronômico do Paraná, PR-Brazil), was used in this study. This strain was cultured in nutrient yeast glycerol (NYG) medium (Daniels *et al.*, 1984) with and without agar addition, at 28 °C. The modified MM1 medium (Schulte and Bonas, 1992) containing 20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4 and 10 mM sucrose was used as the basal medium. For the induction experiments, the bacterium was grown in NYG medium until an A_{600} = 1.2 was reached in order to obtain cellular mass. The cells were centrifuged, resuspended in distilled water and added to the different media (MM1, MM1 + leaf extract and NYG). The initial A_{600} in all the media was of approximately 0.3.

Plant leaf extract preparation

The leaf extract was prepared by triturating 10 g of fresh leaves of sweet orange (*Citrus sinensis*) without the midribs in 100 mL of MM1 medium, resulting in a concentration of 100 mg/mL. The mixture was homogenized (Politron – Superohm) and centrifuged twice at 3800 g for 20 min. The supernatant was recovered, filtered in Millipore 0.2 µm and maintained at -20 °C. For the induction experiments, 1 mL of this leaf extract (100 mg/mL) was added to 100 mL of medium in order to obtain a final leaf extract concentration of 10 mg/mL.

Inoculations of *Citrus sinensis* leaves

For the *in vivo* experiments, the bacterium was grown overnight in NYG medium, centrifuged, washed and resuspended in distilled water and adjusted to an A_{600} = 0.6. *Citrus sinensis* leaves were infiltrated using a syringe and bacterial cells were recovered after 6 days according to Mehta and Rosato (2003) and used for RNA extraction.

RNA preparation and cDNA synthesis

The bacterium was grown for 12 h in the minimal medium MM1, MM1 containing leaf extract of the host plant (10 mg/mL) and in the complex medium NYG. The induction time and leaf extract concentration of the culture media were previously determined by Mehta and Rosato (2001) in the analysis of the differential expression of proteins of *X. axonopodis* pv *citri*. The bacterium was also recovered from host plant leaves 6 days after inoculation. The RNA from each treatment was extracted using 750 µL extraction buffer (1 mM EDTA; 0.1 M Tris HCl; 0.1 M LiCl). The same volume of phenol/chloroform/isoamyl alcohol (25:24:1) containing SDS 1% was added and the suspension centrifuged for 3 min. The supernatant was recovered and the phenol extraction was repeated twice. RNA was precipitated by the addition of 1/20 volume of sodium acetate 40% (w/v) and 2 volumes of ethanol, resuspended in 80µL RNAse free H_2O and treated with DNAse (Gibco). The quality and amount of RNA were verified by agarose gel electrophoresis using a denaturing agarose gel 1%, containing formaldehyde 6% and MOPS buffer 1X. cDNA synthesis was performed using the Time Saver cDNA synthesis kit (Amersham) with some modifications as reported by Bowler *et al.* (1999).

Generation of subtractive libraries by cDNA RDA

The cDNA RDA is a subtractive methodology, in which one cDNA population (driver) is hybridized in excess against a second population (tester), to remove common sequences, consequently enriching for sequences unique to the tester population. In this work, cDNA RDA was performed essentially as described by Bowler *et al.* (1999), with some modifications. cDNA of the bacterium grown in the presence of leaf extract, *in vivo* and in the complex medium was used in independent experiments as tester. In all subtractions, cDNA of the bacterium grown in the minimal medium was used as driver1. Since rRNA is present in high amounts in bacterial RNA preparations, the 16S and 23S genes were also included as driver2. Each cDNA population was digested with *Dpn*II (New England) and adaptors were ligated. After ligation, tester and driver populations were amplified using 30 cycles of 1 min at 95 °C and 3 min at 72 °C for the generation of the representations. All representations were digested with *Dpn*II and new adaptors were ligated only to the tester fragments. Subsequently, a subtractive hybridization was performed with a tester:driver ratio of 1:100, where the ribosomal genes represented half of the amount of driver used (1:50:50 - tester:driver1:driver2). PCR reactions using diluted hybridized DNA were performed to amplify the first difference products (DP1). These products were digested with *Dpn*II, ligated to new adaptors and then used in a second subtractive hybridization using a tester:driver1:driver2 ratio of 1:400:400. The third round of subtractive hybridization was carried out following the same procedure described above using tester:driver1:driver2 ratios of 1:2500:2500 or 1:2500:5000.

DNA sequencing and analysis

The difference products obtained after the second and third subtractive hybridizations were cloned into pGEM T-easy vector (Promega) and the plasmids were purified using the Concert Rapid Plasmid Miniprep System (Gibco). At least 40 fragments from each treatment were sequenced. The sequencing reactions were performed in a total volume of 10 µL containing 800 ng of DNA, 5 pmoles of primer (M13 forward or M13 reverse), 3 µL of ABI PRISM big dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The reactions were conducted with an initial denaturation of 2 min at 95 °C, followed by 25 cycles of 12 s at 95 °C, 6 s at 50 °C and 4 min at 60 °C. The se-

quencing was performed in an automatic sequencer (ABI PRISM™ 377, Perkin Elmer) and the homology of the sequences was searched in the genome of *X. axonopodis* pv. *citri* using the BLAST (Altschul *et al.*, 1990) program.

RT-PCR using specific primers

RT-PCR was performed as previously described (Shepard and Gilmore, 1999). cDNA was synthesized with random primers using the Time Saver cDNA synthesis kit (Amersham) and PCR reactions were performed in a final volume of 25 µL containing 2.0 µL of cDNA, 1.5 mM $MgCl_2$, 125 µM dNTP, 25 pmol of primer and 5 U *Taq* DNA polymerase (Pharmacia). After 5 min denaturation at 95 °C, the amplification was followed by 25 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 74 °C. The amplification products were visualized in 1% agarose gels stained with ethidium bromide. The primers used were designed from the complete sequence of the genes obtained from the genome database of *X. axonopodis* pv. *citri*.

Results and Discussion

cDNA RDA

The cDNA RDA technique was employed for *X. axonopodis* pv. *citri* in an attempt to discriminate genes under regulation of different conditions. The cDNAs of the bacterium recovered directly from infiltrated leaves, grown in medium containing leaf extract or in complex medium were used in 3 rounds of subtractive hybridization against cDNAs of the bacterium cultured in the minimal medium. The cDNA population of each treatment was digested with *Dpn*II, as described for *Neisseria meningitides* (Bowler *et al.*, 1999), however, most representation fragments obtained for *X. axonopodis* pv. *citri* showed smaller sizes (300-800 bp). The difference products obtained were also small and ranged between 100-300 bp, however the actual sizes of the sequences representing the difference products ranged from 70-150 bp due to the presence of concatenated adaptors linked together. The identification of the differentially expressed genes, although generally based on short sequences, was performed successfully since the genome sequence of *X. axonopodis* pv. *citri* was already available (Silva *et al.*, 2002). Another problem encountered was the high amount of ribosomal genes identified in the sequencing step (approximately 30%). In order to reduce the number of clones representing ribosomal genes, the third subtraction in the NYG and *in vivo* conditions was performed using a higher proportion of 16S and 23S representations. A tester:driver1:driver2 proportion of 1:2500:5000 was used and a reduction in the number of 16S and 23S sequences identified was observed (approximately 15%).

According to Bowler *et al.* (1999), the second difference products represent genes, which are more abundantly expressed in a specific treatment, whereas the third difference products reveal genuine differences. Although a third round of subtractive hybridization may reduce the diversity of genes, in the present study, a higher number of fragments obtained after the third hybridization was sequenced in an attempt to identify genes exclusively expressed in the treatments analyzed.

After the subtractive hybridizations of the three treatments (MM1+leaf extract, *in vivo* and NYG) with MM1, the difference products were visualized in agarose gels in a high number as a smear. The difference products obtained after the second and third hybridizations were cloned and sequenced and a total of 37 genes were identified. Overall, the genes revealed belonged to five major functional categories: hypothetical, metabolism, cellular processes, mobile genetic elements and pathogenicity. Out of the 11 genes identified in the leaf extract treatment, 3 encoded hypothetical proteins, 7 represented genes related to metabolism and 1 was associated to pathogenicity (Table 1). In the *in vivo* treatment, a total of 18 genes were identified, including 5 genes encoding hypothetical proteins, 7 genes associated with metabolism, 4 related to cellular processes, 1 mobile genetic element and 1 gene involved in pathogenicity (Table 1). In the NYG medium, out of the 14 genes identified, 6 represented genes encoding hypothetical proteins, 5 genes related to metabolism, 1 to cellular processes, 1 mobile genetic element and 1 gene associated to pathogenicity (Table 2).

RT-PCR using specific primers

In order to confirm the differential expression of the genes identified by cDNA RDA, specific primers for 17 genes revealed in this study were synthesized and used in RT-PCR reactions (Table 3). Also primers for the gene *efp* encoding the elongation factor P protein (XAC1849), which was constitutively expressed in the treatments MM1, MM1+leaf extract and NYG (Mehta and Rosato, 2001) was synthesized and used as a control. The RT-PCR reactions performed herein were not quantitative and therefore only the presence or absence of bands was considered.

The differential expression was confirmed for most genes tested (Figure 1). Only a gene encoding the hypothetical protein (XAC1749) expressed *in vivo* showed an unexpected result with an amplification product from the MM1 treatment. These results reveal a false positive proportion of approximately 6%. The constitutive gene used as a control was expressed in all media.

To investigate whether the genes identified *in vivo* were also expressed in the presence of leaf extract, primers for 5 genes (*flgE*, *yfcB*, *intS*, *nodI* and *pstK*) expressed in the *in vivo* condition were tested using cDNA from the bacterium grown in leaf extract and the amplification product was obtained in all cases (Table 3). Also, cDNA from the bacterium recovered from the plant leaves was used in RT-PCR reactions with the primers designed for the genes *atpD*, *nuoH* and *tlyC*, all differentially expressed in the leaf extract condition. The results revealed the amplification

Table 1 - Differentially expressed genes, regulated in the presence of leaf extract and *in vivo* detected by cDNA RDA.

DP[1]	Homology	Gene	ORF
	MM1 + leaf extract of *Citrus sinensis* against MM1		
	Hypothetical proteins		
DP3	Conserved hypothetical protein	none	XAC0744
DP3	Conserved hypothetical protein	none	XAC4181
DP3	Conserved hypothetical protein[2]	none	XAC0289
DP3	Conserved hypothetical protein	none	XAC0259
	Macromolecule metabolism		
DP3	Peptidyl-dipeptidase[2]	*dcp*	XAC0249
	Intermediary metabolism		
DP3	Oxidoreductase[2]	*mocA*	XAC0288
DP2	ATP synthase, beta chain	*atpD*	XAC3649
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[2]	*nuoL*	XAC2693
DP3	NADH-ubiquinone oxidoreductase, NQO8 subunit	*nuoH*	XAC2697
	Cellular processes/transport		
DP2	HPr kinase/phosphatase	*pstK*	XAC2975
DP2	Cell division protein	*ftsY*	XAC2552
DP3	Solute:Nag + symporter	*ppa*	XAC4176
	Pathogenicity, virulence and adaptation		
DP2	Hemolysin	*tlyC*	XAC1709
	In vivo against MM1		
	Hypothetical proteins		
DP3	Conserved hypothetical protein[2]	none	XAC0289
DP2	Conserved hypothetical protein	none	XAC2902
DP3	Conserved hypothetical protein	none	XAC4181
DP3	Conserved hypothetical protein	none	XAC0376
DP3	Conserved hypothetical protein	none	XAC0357
DP3	Conserved hypothetical protein	none	XAC1749
	Macromolecule metabolism		
DP2	60 KDa chaperonin	*groEL*	XAC0542
DP2	Adenine-specific methylase	*yfcB*	XAC2726
DP3	Peptidyl-dipeptidase[2]	*dcp*	XAC0249
DP3	50S ribosomal protein L5	*rplE*	XAC0984
	Intermediary metabolism		
DP3	Oxidoreductase[2]	*mocA*	XAC0288
DP2	Histidine kinase/response regulator hybrid protein	none	XAC0685
DP2	Transcription regulator	none	XAC1311
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[2]	*nuoL*	XAC2693
	Cellular processes/transport		
DP2	TonB-dependent receptor	*btuB*	XAC1310
DP3	ABC transporter ATP-binding protein	*nodI*	XAC1547
DP3	Flagellar biosynthesis, hook protein	*flgE*	XAC1983
DP3	HPr kinase/phosphatase	*pstK*	XAC2975
	Mobile genetic elements		
DP3	Phage-related integrase	*intS*	XAC2286
	Pathogenicity, virulence and adaptation		
DP2	Sequence between rpfF and rpfB	*rpfF/rpfB*	XAC1879/XAC1880

[1]Difference product: DP2 indicates products from the second hybridization and DP3 from the third.
[2]Differentially expressed sequences in all treatments identified by cDNA RDA.

Table 2 - Differentially expressed genes, regulated in the complex medium NYG detected by cDNA RDA.

DP[1]	Homology	Gene	ORF
	NYG against MM1		
	Hypothetical proteins		
DP2	Conserved hypothetical protein	none	XAC2444
DP2	Conserved hypothetical protein	none	XAC2902
DP2	Conserved hypothetical protein	none	XAC2925
DP3	Conserved hypothetical protein	none	XAC1372
DP3	Conserved hypothetical protein[2]	none	XAC0289
DP3	Conserved hypothetical protein	none	XAC0259
DP3	Hypothetical protein	none	XAC3025
	Macromolecule metabolism		
DP2	50S ribosomal protein L21	*rplU*	XAC1248
DP3	Peptidyl-dipeptidase[2]	*dcp*	XAC0249
DP3	Endonuclease precursor	*nucA*	XAC3769
	Intermediary metabolism		
DP3	Oxidoreductase[2]	*mocA*	XAC0288
DP3	Two-component system regulatory protein	none	XAC0684
DP3	NADH-ubiquinone oxidoreductase, NQO12 subunit[2]	*nuoL*	XAC2693
	Cellular processes/transport		
DP2	Solute:Na+ symporter	*ppa*	XAC4176
	Mobile genetic elements		
DP3	ISXac 3 transposase	*ISxac3*	XAC0091
	Pathogenicity, virulence and adaptation		
DP3	CAP-like protein	*clp*	XAC0483

[1]Difference product: DP2 indicates products from the second hybridization and DP3 from the third.

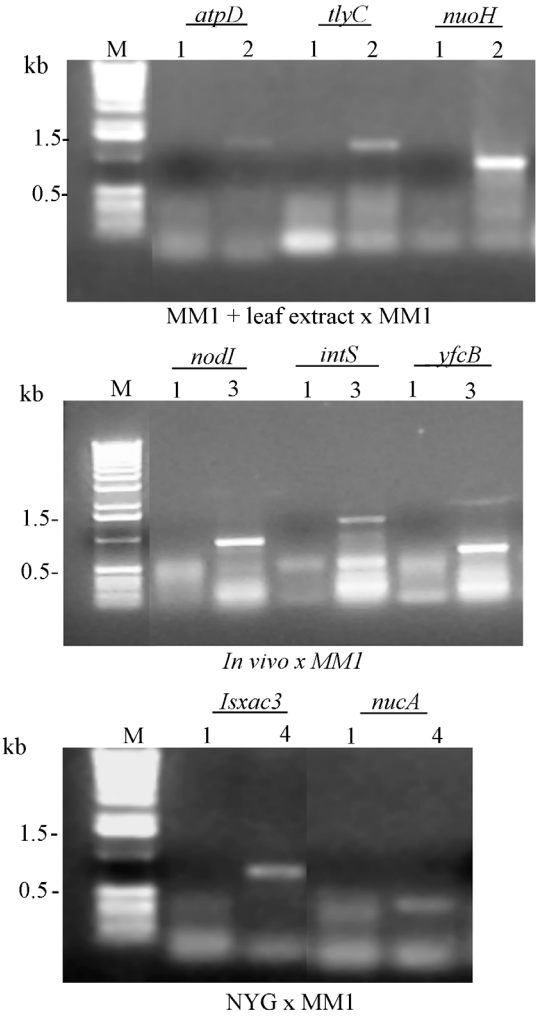

Figure 1 - RT-PCR using cDNA of *X. axonopodis* pv. *citri* grown in: 1, MM1; 2, MM1 + leaf extract of *Citrus sinensis*; 3, *in vivo* and 4, NYG. The reactions were performed using primers for the genes *atpD* [1349 bp], *tlyC* [1290 bp], *nuoH* [899 bp], *nodI* [814 bp], *intS* [1200 bp], *yfcB* [600 bp], *ISxac3* [762 bp] and *nucA* [371 bp], as indicated. M, molecular size marker Ladder 1kb [Gibco].

product for the first two genes, but not for hemolysin (Table 3). The comparison of the use of leaf extract of the host plant with *in vivo* conditions revealed that the use of the leaf extract may be considered a valid method mimicking the *in vivo* environment, since several differentially expressed genes were detected in both treatments.

Primers for the genes *atpD*, *nuoH, tlyC*, *yfcB*, *flgE*, *pstK*, *intS* and *nodI*, differentially expressed *in vivo* and in the presence of leaf extract were also tested using cDNA from the NYG medium and an amplification product was revealed only when primers for *nuoH* (ABC transporter) and *nodI* (NADH ubiquinone oxidoreductase, NQO8 subunit) were used (Table 3). These results indicate that the other genes are not expressed in the rich medium and may be specifically induced by components present in the leaf tissue.

Differentially expressed genes

Genes with unassigned function. A high number of genes found in the present work represented genes that encode hypothetical proteins (31%). This finding is not surprising since in *E. coli*, one of the most studied bacterium, similar results have been obtained when comparing the expression in minimal and complex media (Tao *et al.*, 1999). Studies with pathogenic bacteria have emphasized mostly the major pathogenicity related genes and therefore a high number of genes with unknown function are observed. Indeed, in *X. axonopodis* pv. *citri*, hypothetical proteins represent approximately 37% of the total genome (Silva *et al.*, 2002). In this study, genes with unassigned function were identified in all treatments analyzed and these genes may play an important role in metabolism or in the plant-pathogen interaction since they were regulated in response to the specific treatments. Interestingly, one hypothetical protein (XAC4181) appeared several times (10) in the sequencing step in the leaf extract and *in vivo* treatments, in-

Table 3 - RT-PCR results for the 17 selected genes using cDNA of the bacterium grown in MM1, MM1 + leaf extract, *in vivo* and in NYG.

Differential product	ORF	Growth conditions			
		MM1	MM1 + leaf extract	*In vivo*	NYG
Conserved hypothetical protein	XAC 4181	-	+	+	
Conserved hypothetical protein	XAC 0289	-	+	+	+
Oxidoreductase	XAC 0288	-	+	+	+
ATP synthase, beta chain	XAC 3649	-	+	+	-
NADH-ubiquinone oxidoreductase, NQ O8 subunit	XAC 2697	-	+	+	+
HPr kinase/phosphatase	XAC 2975	-	+	+	-
Hemolysin	XAC 1709	-	+	-	-
Conserved hypothetical protein	XAC 1749	+		+	
60 KDa chaperonin	XAC 0542	-		+	
Adenine-specific methylase	XAC 2726	-	+	+	-
50S ribosomal protein L5	XAC 0984	-		+	
ABC transporter ATP-binding protein	XAC 1547	-	+	+	+
Flagellar biosynthesis, hook protein	XAC 1983	-	+	+	-
Phage-related integrase	XAC 2286	-	+	+	-
Endonuclease precursor	XAC 3769	-			+
ISXac 3 transposase	XAC 0091	-			+
CAP-like protein	XAC 0483	-			+

The symbols + and - indicate presence and absence of amplification, respectively.

dicating that this gene may be highly expressed in these treatments.

Genes expressed in leaf extract, *in vivo* and NYG conditions. In the present study, several genes involved in metabolism and transport were expressed in all treatments analyzed. Among these differentially expressed sequences were genes that encode a peptidyl dipeptidase, which removes dipeptides from the C terminal of substrates and NADH-ubiquinone oxidoreductase, NQO12 and NQO8 subunits, which constitute a proton pump considered the first complex of energy transduction of several respiratory chains (Scheide *et al.*, 2002). A sequence between two genes that encode a hypothetical protein (XAC0289) and an oxidoreductase associated with electron transport was also obtained in all conditions. Another gene involved in the acquisition of nutrients identified in all treatments was an ABC transporter. These transporters are energy-dependent membrane proteins that translocate a variety of substrates such as biotin, sulfate, maltose, ribose, phosphate, among others, through the cellular membrane (Mishima *et al.*, 2001). ABC transporters have been considered important for phytopathogenic bacteria to grow *in planta* (Llama-Palacios *et al.,* 2002), probably due to their involvement in the transport of sugar and amino acids present in the plant tissue. Many of these genes may have been differentially expressed due to the difference in the presence of nutrients in the media used. The minimal medium MM1, used as a control is poor in nutrients and therefore the bacterium grows slowly, when compared to the other culture conditions (Mehta and Rosato, 2001). Peptides and amino acids, for example, are essential growth factors for several bacteria and enzymes such as peptidyl dipeptidase should be important for the degradation of proteins which are more abundant in the leaf extract, NYG and probably *in vivo* treatments.

Genes involved in metabolism/transport/cellular processes. Some genes were identified in specific treatments and included genes encoding ribosomal proteins, which are also highly expressed by fast growing bacterial cells (Karlin and Mrázek, 2000). Two genes that encode ribosomal proteins L21 and L5 were differentially expressed in NYG and *in vivo*, respectively. Protein L21 ligates to the 23S rRNA in the presence of protein L20 and L5 is one of the proteins that mediates the ligation of the 5S RNA subunit to the large ribosome subunit and has an important role in the conformation of the 5S rRNA. Karlin and Mrázek (2000) report that for rapid division, many ribosomes are indispensable, augmented by abundant transcription processing factors and chaperones needed to assure properly translated, modified and folded protein products. A chaperone (GroEL) was also differentially expressed in the *in vivo* condition. These proteins contribute to conformational changes and to minimize protein damage during stress.

Other genes differentially expressed associated with metabolism were an adenine specific methylase in the *in vivo* treatment and an endonuclease precursor in the NYG condition. Both genes are related to DNA and RNA modification and play a role in the regulation of gene expression. The adenine specific methylase recognizes a sequence of 4-8 nucleotides and modifies the nucleotide inside the sequence. This enzyme also has an important role in the DNA replication, methyl-directed mismatch repair, transposition and gene expression (Radlinska *et al.*, 2001). The endonuclease catalyzes the degradation of DNA and RNA, and in general has an effect over the stability of the RNA and therefore also on the levels of translation.

Several transport genes, involving Na and Fe, were also identified in the treatments analyzed. The solute/Na+ symporter, expressed in the presence of leaf extract and NYG, uses free energy from the electrochemical gradients of Na+ to accumulate solutes (Jung, 2001). In *E. coli* for example, the transport of melobiose, proline, pantothenate and glutamate is coupled to Na+. The majority of transporters from this family are involved in the acquisition of nutrients and others are associated with osmoadaptation (Jung, 2001). The membrane energetics based on Na+ has several advantages such as the increase of the versatility of the pathogen by providing an additional form of ATP synthesis, motility and solute transport. These factors increase the chances of colonizing the host cell and survival of the pathogen in the host organism (Hase *et al.*, 2001).

Another differential sequence obtained was localized between two genes, one of them associated with the transport of Fe (TonB dependent receptor) and the other was identified as a transcriptional regulator. TonB is associated with the transport of Fe, and has a crucial role in the plant-pathogen interactions (Expert *et al.*, 1996; Loper and Buyer, 1991). Fe is the limiting factor of bacterial growth *in planta* since micromolar concentrations of iron are necessary to permit bacterial growth and multiplication. The TonB system also mediates the signal transduction from the cellular surface to the cytoplasm, as reported in *E. coli* and *Pseudomonas putida* (Harle *et al.*, 1995; Koster *et al.*, 1994). In *X. campestris*, the genes involved in the uptake of Fe are essential for the induction of a hypersensitive response (Wiggerich and Puhler, 2000).

At this stage, it is difficult to know the specific role played by these genes. It is possible that they are associated with the intense bacterial growth in the different culture conditions (presence of leaf extract, *in vivo* and NYG) when compared to the slow growing cells in the minimal medium MM1. Intense bacterial growth is also considered essential for plant colonization and therefore for invasion of plant tissue. Although the genes differentially expressed in the presence of leaf extract and *in vivo*, are directly involved in metabolism, they may also play a role in pathogenicity by regulating the level and time of the appearance of plant symptoms.

Two-component systems. The signal transduction systems have been extensively studied in Gram negative bacteria and can influence bacterium-host interactions. These systems involve a signal transduction of a sensor protein, such as histidine kinase, to a transcriptional regulator of several genes and are essential for the adaptation of bacteria to stress and environmental changes (Matsushita and Janda, 2002). Bacteria also use these systems to secrete several proteins into the extracellular environment. In the present study, genes involved in two-component systems were identified in all three treatments analyzed. In the leaf extract and *in vivo* treatments the HPr kinase/phosphatase gene was differentially expressed. Also a histidine kinase/response regulator hibrid protein was differentially expressed *in vivo*. It is possible that components *in planta* and in the leaf extract induced the expression of these genes allowing the bacterium to sense the new environment. A two-component regulatory protein was also identified in the NYG medium. Similar results were obtained in *Erwinia amylovora* where putative two-component response regulators were expressed in rich media (Wei *et al.*, 2000).

A protein related to flagellum biosynthesis, the hook protein, was also differentially expressed *in vivo*. Although the flagellum is classified as related with metabolism and chemotaxis, flagellar proteins have been associated with efficient plant colonization (Nasser *et al.*, 2001). For the assembly of the flagellum, several protein subunits are exported from the cytoplasm to the outer surface of the cell by a mechanism, which is similar to the type III secretion system (Young *et al.*, 1999). This system has been well studied in phytopathogenic bacteria and several proteins involved in this mechanism share homology to proteins associated with the biosynthesis of the flagellum. In *E. coli*, the flagellar export apparatus also functions as a protein secretion system and in several bacteria it has been considered essential for bacterial viability (Young *et al.*, 1999). Moreover, it has been reported that the flagellum biosynthesis occurs in response to environmental signals and therefore the regulation of the synthesis of the flagellum can influence bacterium-host interactions independent of motility (Young *et al.*, 1999).

Mobile genetic elements. An insertion sequence (ISXac3) was differentially expressed in the NYG medium. This insertion sequence belongs to the IS3 family, which is highly representative in *X. axonopodis* pv. *citri*. Twenty-one copies of ISXac3 were identified in the genome of this bacterium (da Silva *et al.*, 2002), however the expression of this gene had not been reported before. Insertion sequences have been related to mutation, and the expression of these genes may be associated with bacterial growth. Studies in *X. oryzae* pv. *oryzae* revealed that spontaneous mutants deficient for virulence and extracellular polysaccharides accumulate in the stationary phase. Results of these studies showed that these mutations occur due to insertion sequences (IS) (Rajeshwari and Sonti, 2000). After 12 h growth in NYG, *X. axonopodis* pv. *citri* is close to the stationary phase (Mehta and Rosato, 2001), which could explain the expression of the transposon ISXac3 in this treatment. In *X. axonopodis* pv. *citri* more than 100 mobile genetic elements were identified (da Silva *et al.*, 2002), and further studies on the factors influencing the activation of these elements would be important to understand the mechanism of mutation caused by transcription events and generation of genetic diversity.

A phage-related integrase was another mobile genetic element identified in the *in vivo* condition. Integrases, besides permitting the integration of sequences in specific sites of the chromosome, may also play an indirect role in

pathogenicity by changing the expression of pathogenicity related genes. In *Vibrio cholera*e, for example, the avirulence gene cluster is associated to an integrase (Kovach *et al.*, 1996). Integrases have also been associated to antibiotic resistance (Oh *et al.*, 2002) as well as integration of pathogenicity islands (Tauschek *et al.*, 2002).

Pathogenicity genes. Among the pathogenicity genes identified in the present work is the gene that encodes hemolysin, which was differentially expressed by the bacterium cultured in the presence of leaf extract. Hemolysin is a glycolipid synthesized by bacteria in the stationary phase in conditions where there is a prevalence of carbon sources over nitrogen sources in the medium (Denisov *et al.*, 1996). Hemolysin has been considered a virulence factor in bacteria such as *Aeromonas*, due to its hemolytic properties and enterotoxic activities (Nomura, 2001). In *Pseudomonas putida*, hemolysin was considered important for the colonization of the rhizophere of several economically important plants (Espinosa-Urgel *et al.*, 2000). In phytopathogenic bacteria, the function of this enzyme is not well established. Studies with *Erwinia chrysanthemi* revealed that the flanking regions of the *hrpC* and *hrpN* genes, involved in the hypersensitive reaction in tobacco leaves, contain homologs of hemolysin (Kim *et al.*, 1998).

Another sequence identified in the *in vivo* treatment was an intergenic region between the *rpf*F and *rpf*B genes, which are involved in pathogenicity. The *rpf* ("regulation of pathogenicity factors") genes control the production of pathogenicity factors such as enzymes and extracellular polysaccharydes (Dow *et al.*, 2000). The *rpf* cluster is formed by at least 7 *rpf*A-G genes (Tang *et al.*, 1991) and studies have shown that mutations in these genes decrease the production of extracellular enzymes and reduce virulence (Barber *et al.*, 1997).

The *rpf*F and *rpf*B genes are involved in the regulation of the synthesis of a diffusible extracellular factor of low molecular weight called DSF ("diffusible signal factor"), which is involved in the production of protease, endoglucanase and polygalacturonate liase (Barber *et al.*, 1997). It is assumed that DSF is a fatty-acid derivative, used by several Gram-negative bacteria for intercellular signalling and regulation. This regulatory system based on a small molecule seems to be essential for pathogenicity.

Unexpectedly, a CAP-like protein ("catabolite activator protein") was differentially expressed by the bacterium grown in the complex medium. This protein regulates directly or indirectly genes implicated in pathogenicity, which are usually repressed in complex media in *Xanthomonas* (Schulte and Bonas, 1992; Wei *et al.*, 1992). de Crecy-Lagard *et al.* (1990) analyzed a mutant of *X. campestris* pv. *campestris* for the CAP-like protein and observed the differential expression of several genes including those involved in the production of xanthan gum, pigment and extracellular enzymes.

In the present study we have reported the analysis of the differential expression of *X. axonopodis* pv. *citri* by cDNA RDA and several genes more abundantly or exclusively expressed in specific treatments were identified. The results obtained herein indicate that cDNA RDA is a rapid and efficient method for the analysis of the differential gene expression of bacteria grown in different culture conditions, and has the advantage of eliminating most of the ribosomal RNA, which is usually a drawback in several techniques such as Differential Display. In the present study, a total of 37 distinct genes regulated by specific environmental conditions were identified. Since the sequencing of the difference products were not exhaustive, it is possible that the isolation of a higher number of these products from each treatment may lead to the identification of additional genes.

The majority of the genes were classified as encoding hypothetical proteins (31%) or related to metabolism (34%). It is likely that macromolecule and energy metabolism genes are induced in the fast growing bacterial cells in the treatments analyzed, however a few genes could be related specifically to the interaction with the citrus plant (presence of leaf extract or *in vivo*) and could be involved in pathogenicity. Among the potential genes associated with the plant-pathogen interaction are those involved in the signal transduction system and transcription regulation that could act in a cascade controlling the expression of different genes. Genes involved in cellular processes and transport such as those associated with flagellum biosynthesis and Hrp kinase/phosphatase are potential candidates for further studies related to pathogenicity. The solute Na+ symporter and TonB receptor genes seem to be regulated by specific conditions but may be more related to the acquisition of inorganic elements than directly in pathogenicity. The category of hypothetical proteins is a great challenge since they are found in high percentages in all bacterial genomes. Several of these genes are regulated by specific conditions suggesting that they can play a significant role in the bacterium life cycle.

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